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THE EFFECT OF REPEATED LOADING AND FREEZE-THAW CYCLING ON IMMATURE BOVINE THORACIC MOTION SEGMENT STIFFNESS

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Abstract:

There is growing interest in the biomechanics of 'fusionless' implant constructs used for deformity correction in the thoracic spine, however, there are questions over the comparability of *in vitro* biomechanical studies from different research groups due to the various methods used for specimen preparation, testing and data collection. The aim of this study was to identify the effect of two key factors on the stiffness of immature bovine thoracic spine motion segments: (i) repeated cyclic loading and (ii) multiple freeze-thaw cycles, to aid in the planning and interpretation of *in vitro* studies. Two groups of thoracic spine motion segments from 6-8 week old calves were tested in flexion/extension, right/left lateral bending, and right/left axial rotation under moment control. Group (A) were tested with continuous repeated cyclic loading for 500 cycles with data recorded at cycles 3, 5, 10, 25, 50, 100, 200, 300, 400 and 500. Group (B) were tested after each of five freeze-thaw sequences, with data collected from the 10th load cycle in each sequence. Group A: Flexion/extension stiffness reduced significantly over the 500 load cycles (-22%; *P*=0.001), but there was no significant change between the 5th and 200th load cycles. Lateral bending stiffness decreased significantly (-18%; *P*=0.009) over the 500 load cycles, but there was no significant change in axial rotation stiffness *(P*=0.137). Group B: There was no significant difference between mean stiffness over the five freeze-thaw sequences in flexion/extension *(P*=0.813) and a near significant reduction in mean stiffness in axial rotation (-6%; *P*=0.07). However, there was a statistically significant increase in stiffness in lateral bending (+30%; *P*=0.007). Comparison of *in vitro* testing results for immature thoracic bovine spine segments between studies can be performed with up to 200 load cycles without significant changes in stiffness. However, when testing protocols require greater than 200 cycles, or when repeated freeze-thaw cycles are involved, it is important to account for the effect of cumulative load and freeze-thaw cycles on spine segment stiffness.

Keywords: Bovine; in vitro biomechanical testing; calf; freeze-thaw cycle; cyclic loading; stiffness; thoracic spine.

Introduction:

There is increasing interest in the biomechanical mechanisms of so-called ‘fusionless’ implants for the correction of thoracic spinal deformities in the growing spine. Owing to the unavailability of paediatric human tissue, in vitro biomechanical tests of fusionless spinal implants rely on animal models, notable the immature bovine spine which has been shown to be a good model for the young human spine. However, as has been shown in lumbar spine biomechanics studies[1-4], care is required when comparing results from different studies, as they often use different specimen preparation, testing and data collection methods[5-7]. Of these methods, the number of load cycles performed prior to recording the data and the number of freeze-thaw sequences that the spine segments were subjected to prior to testing (occurring when the same specimens are repeatedly tested on different days) vary considerably in the literature[7-12]. To date, there is a paucity of information on the effect of loading and/or specimen storage protocols on the biomechanical properties of thoracic motion segments. Therefore, the aims of this study were to identify the effect of repeated cyclic loading on immature bovine thoracic spine segment stiffness, and to quantify the effect of multiple freeze-thaw sequences.

Materials and Methods:

Specimen Preparation

Thoracic spines from 6-8 week old calves weighing 40-60kg were obtained from a local abattoir. These were frozen fresh and kept at -20°C in double plastic bags until testing. The thawing process was adopted from a previous study[13], keeping them for 24 hours in a refrigerator regulated at +2°C (±1°). They were then left at room temperature (21±2°C) for 4 hours. Each spine was then dissected maintaining the full length of the spinous process and 5cm of attached rib length bilaterally. The attached muscles were removed with care to avoid any damage to bony or ligamentous structures. The spines were divided into individual motion segments, consisting of two vertebrae and an intervertebral disc with attached ligaments and rib heads. There were four specimens of each of the motion segments: T4/5, T5/6, T6/7, T7/8, T8/9, T9/10 and T10/11 (n=28). Each segment was potted in polymethylmethacrylate with 3 screws inserted in the endplates above and below to improve fixation. The segments were then positioned in an environmental chamber at 37±2°C and 100% humidity by being loosely wrapped in gauze and periodically sprayed with 0.9% saline (phosphate buffered) for 1 hour. This was shown to bring the intervertebral disc temperature to 34°C.

Testing Schedule

Testing was conducted with the specimens in the environmental chamber[2]. An Instron biaxial materials testing machine (Instron, 8874, Norwood, Massachusetts, USA) was fitted with a custom made spine testing jig which allowed free x-y horizontal plane movement via high precision linear bearings at the base (Figure 1).

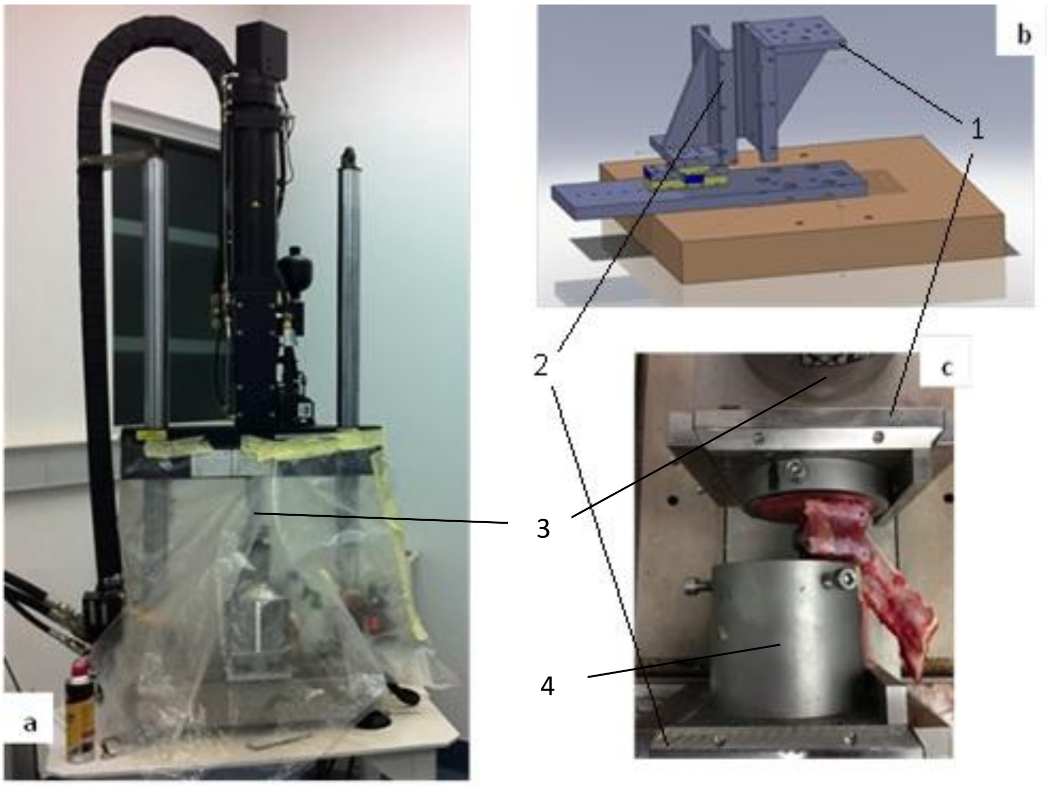

Figure 1: a) Instron Biaxial materials testing machine, b) Custom made testing jig with free horizontal plane movement. The diagram shows the jig set up for flexion/extension or lateral bending as plate (1) attaches to the vertical testing shaft of the Instron and the other plate (2) attaches to the caudal anchoring plate on the free x-y movement jig, c) Example of test specimen in jig set up to test flexion/extension, (3) represents the vertical Instron testing shaft which rotates to provide flexion/extension, (4) shows an extension tube used to allow clearance of the spinous process.

A pilot study was performed to determine the moment required to produce a rotation of ± 6° in each of the three primary axes (6° being an average range of movement in human thoracic spines[14]) at a rate of 1 deg/sec^{-1} (Table 1). [2] Fourteen segments were tested with the average required moment to produce ±6° rotation being ±1.73N°m for flexion/extension, ±1.05 N°m for lateral bending and ±1.27 N°m for axial rotation. These values were then used in the main study. The force required to overcome static friction in the x-y bearing plate was measured to be 0.49N. This resulted in a frictional moment of 0.12 N°m in the flexion/extension and lateral bending modes. In axial rotation the frictional moment was negligible.

Table 1: Pilot study results used to determine moment in each plane of motion required to achieve approximately ±6 degrees of rotation for subsequent tests.

Direction of Movement	Mean Moment (Nm)	Minimum Moment (Nm)	Maximum Moment (Nm)	Standard Error (Nm)
Flexion/Extension	1.73	1.25	2.45	0.086
Lateral Bending	1.05	0.35	1.78	0.114
Axial Rotation	1.27	0.69	2.09	0.1

Based on the pilot study, the 28 specimens in the main study were tested using moment control to the above values at a linearly ramped loading/unloading rate of 0.3 N°m per second; in order give a similar loading rate to that of the pilot study. Moment and rotation data for the primary loading axis were logged at 100Hz during testing.

The 28 segments were divided into two equal groups, each containing 14 motion segments:

- Group (A) were tested with continuous reversed cyclic loading for 500 cycles with data recorded at cycles 3, 5, 10, 25, 50, 100, 200, 300, 400 and 500.
- Group (B) were tested with 10 load cycles after each of 5 freeze thaw sequences. After each testing cycle the segments were frozen again at -20°C for a minimum of 24 hours and subsequently thawed using the same protocol described previously. None of the segments were tested prior to initial freezing. The first testing point always occurred after the first freeze/thaw cycle. Data were collected from the tenth load cycle of each sequence.

The choice of 14 segments per study was based on the prior work of Shillington et al[6], who found that a 10% change in the stiffness of immature bovine spinal segments could be detected with a power of 0.8 using n=14 specimens.

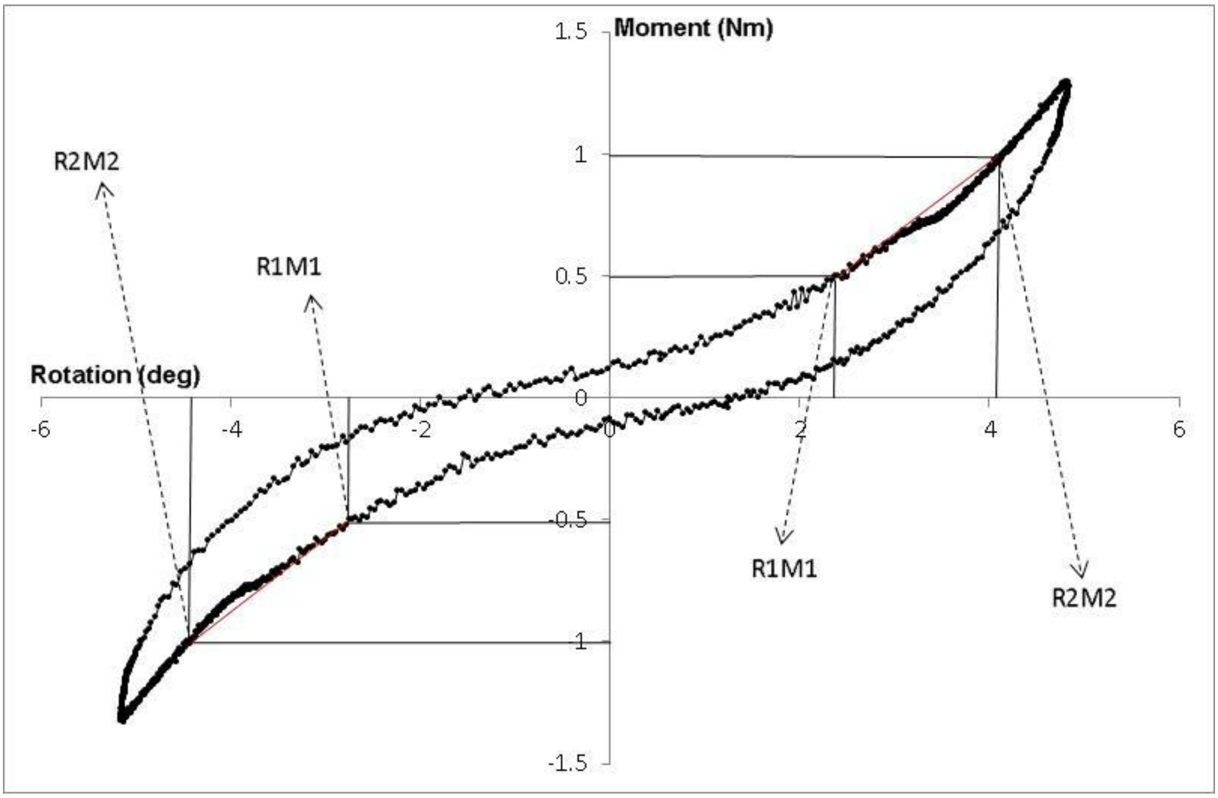

Figure 2: Typical moment versus rotation Curve indicating points for stiffness calculation. R1M1= Rotation 1, Moment 1; R2M2= Rotation 2, Moment 2

Statistical Analysis

Stiffness was calculated from the moment versus rotation curve between ±0.5 Nm and ±1Nm (Figure 2: Stiffness = (Moment2-Moment1)/(Rotation2-Rotation1)). Mean, standard deviation, median, mode, standard error of mean, minimum and maximum stiffness were calculated for each data collection cycle. Data from both groups were tested for normality using the Kolmogorov-Smirnov test. Statistical analyses of the data were performed using SPSS (version 21.0, IBM, Armonk, NY) generalised linear models for repeated measures ANOVA with pairwise testing using Bonferroni correction. A significance level of $P<0.05$ was considered statistically significant. For each test, motion segment stiffness (k) was normalised to the initial value (k0) to allow graphing of changes in stiffness ratio (k/k0) over the course of testing.

Results:

Effect of Multiple Load Cycles: (Figure 3)

Flexion/Extension: There was a 22% (*P*=0.001) reduction in mean motion segment stiffness in flexion/extension after 500 cycles compared with the initial (3rd cycle) stiffness of 0.39 Nºm deg^{-1}, as shown in Figure 3a. Further pairwise tests showed that a statistically significant difference was only evident after the 50th cycle. However, using the 5th (rather than the 3rd) cycle as a baseline increased the number of cycles needed to produce a significant difference to the 200th cycle. The decrease in stiffness with increasing number of cycles was equally apparent in both flexion and extension as shown in Figure 3d.

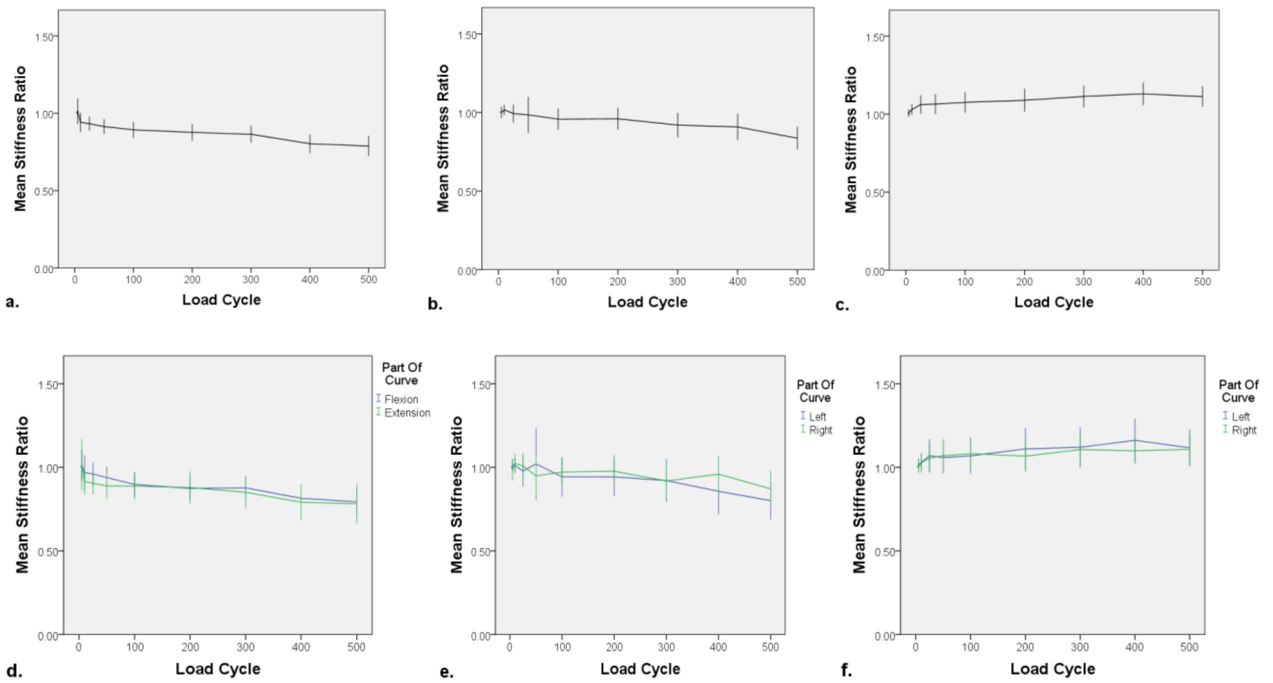

Figure 3: Mean stiffness ratios (k/k0) vs. load cycle number. a: Flexion/extension, b: Lateral bending, c: Axial rotation. d: Demonstrating flexion and extension separately, e: Demonstrating left and right lateral bending individually and f: Demonstrating left and right axial rotation separately. Error bars indicate 95% confidence interval. k0 is taken from the 3rd load cycle

Right and left lateral bending: As shown in Figure 3b, there was also a significant reduction in motion segment stiffness in lateral bending with increasing number of load cycles, such that stiffness reduced by 18% after 500 cycles compared to the initial (3rd cycle) value of 0.31Nm deg^{-1}

(*P*=0.009). Pairwise tests showed the difference to be insignificant up to the 400th load cycle, but then there is an 8% drop in stiffness between the 400th and 500th cycles, which makes the overall stiffness change become statistically significant. In contrast to the statistically significant change in flexion/extension stiffness between the 5th and 200th cycles, the 5% drop in lateral bending stiffness between these cycles was not statistically significant.

Right and left axial rotation: There was no significant change in motion segment stiffness in axial rotation with increasing number of load cycles (see Figure 3c). From an initial value of 0.29 Nm deg^{-1}, there was a total increase of 9% in the mean motion segment stiffness over 500 load cycles, however, this was not statistically significant (*P*=0.137). In contrast to the statistically significant change in flexion/extension stiffness between the 5th and 200th cycles, the 6% increase in axial rotation stiffness between these cycles was not statistically significant.

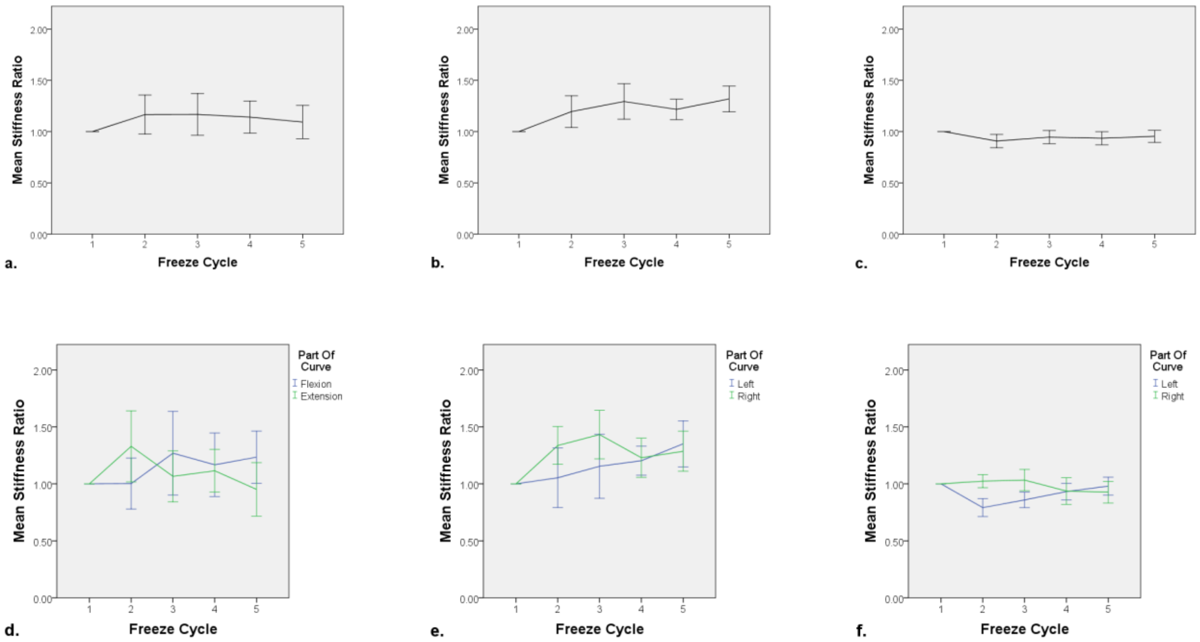

Figure 4: Mean stiffness ratios (k/k0) relative to freeze-thaw cycle number. a. Flexion, extension, b. Lateral bending, c. Axial rotation, d. Demonstrating flexion and extension separately, e. Demonstrating left and right lateral bending separately, f. Demonstrating right and left axial rotation separately. Error bars indicate 95% confidence interval. k0 is taken from the 3rd load cycle.

Effect of Multiple Freeze Thaw Cycles: (Figure 4)

Flexion/Extension: There was no statistically significant change in motion segment stiffness in flexion/extension between freeze-thaw cycles one and five from the initial stiffness value of 0.27

Nm deg^{-1} (P=0.813). However, on further analysis of the individual effects on flexion and extension, there was no significant change in flexion stiffness (P=0.336) but a significant (P=0.041) 9% decrease in extension stiffness (Figure 4d); as the graph shows there was an initial (non-significant) increase in extension stiffness between the 1st and 2nd freeze cycles of 23% (P=0.114).

Right and left lateral bending: As shown in Figure 4b, there was a statistically significant increase in motion segment stiffness in lateral bending from the initial value of 0.2 Nm deg^{-1}, with repeated freeze-thaw cycles, of 26% (P=0.003). There was no statistically significant difference between left and right lateral bending stiffness (P= 0.598). However between the 1st and 2nd freeze cycles there was a 20% increase in stiffness which was near significant (P=0.088). This was followed by a transient drop in stiffness of 5% over the 3rd and 4th freeze/thaw cycles, followed by a 10% increase. Neither of these changes were statistically significant however.

Right and left axial rotation: There was no statistically significant change in motion segment stiffness with repeated freeze-thaw cycles (see Figure 4c); however the P-value of 0.07 was near-significant, with a mean reduction in motion segment stiffness of 6% relative to the initial value of 0.28 Nm deg^{-1}. The largest reduction occurred between the 1st and 2nd freeze cycles of 9% which was statistically significant (P= 0.04). Following this there was a mild increase in segment stiffness over the subsequent freeze/thaw cycles of 4% which was not statistically significant. There was no statistically significant difference between left and right axial rotation (P=0.111).

Discussion

Owing to increasing interest in spinal pathophysiology and 'fusionless' treatment methods for paediatric deformity, and due to their similarity to human spines, immature bovine (calf) spines have been previously suggested as an appropriate model for the young human spine[7, 15-18], and were chosen for the current study. Aside from differences between research groups in the design of the spine testing equipment used, another important potential source of variability between and within studies is the testing protocol itself, particularly with regard to the number of times a particular motion segment is loaded (i.e. the cumulative number of loading cycles)[6, 12, 18], and also whether the same motion segment is tested on successive days by freezing and re-thawing. The current study, therefore, characterised the effects of these two testing variables on motion segment stiffness in the immature bovine thoracic spine.

The results showed statistically significant decreases in motion segment stiffness in two of the three loading directions (22% decrease in flexion/extension, 18% decrease in lateral bending, no significant change in axial rotation) between the 3rd and 500th load cycles, and a relatively large (30%) statistically significant increase in lateral bending stiffness after 5 freeze-thaw cycles, but no statistically significant changes in combined flexion/extension or axial rotation. These results suggest that calf thoracic spine segments can be tested for up to 200 cycles with minimal change in stiffness due to multiple cycling, but beyond 200 cycles caution should be used in interpreting the results as the changes in stiffness are appreciable.

To the best of our knowledge there has only been one previous study examining the effect of repeated cycling on motion segment stiffness, and this was performed in axial rotation only on mature sheep spines, loaded to $\pm$5Nm to a maximum of 500 loading cycles[2]. This prior study did not find any change in axial rotation stiffness, which agrees with the finding of our current study for axial rotation.

As for the effect of repeated freeze-thaw cycles, a recent study by Tan and Uppuganti [4] found that flexibility increased (reduced stiffness) in motion segments of a mature (elderly) human cadaver lumbar spine. This was tested with a moment of $\pm$7.5Nm in all primary directions of flexion/extension, lateral bending and axial rotation with measurements taken at the fifth cycle. Hongo et al[3] explored the effect of freeze thaw cycles on the biomechanics of porcine lumbar motion segments under an applied moment of $\pm$5Nm with three freeze-thaw cycles. These authors found that after the initial freeze there was no significant change for subsequent freeze-thaw cycles. Although our findings agree with this in flexion/extension and axial rotation, we found a difference between the effects of repeated freeze-thaw cycles on flexion and extension stiffness when the two loading directions are assessed individually. Flexion stiffness was not significantly affected; however extension stiffness was affected. In lateral bending, our results run counter to those of both previous studies, as a significant increase in stiffness was recorded. However we note that the elderly human lumbar spine tested in Tan and Uppuganti et al may have been more prone to damage than the healthy immature bovine thoracic spines used in the current study. As is shown in Figure 4, the changes in stiffness that occurred between the first and second freeze/thaw cycles were often larger than the changes occurring between pairs of subsequent freezing cycles, however these 1st to 2nd freeze cycle changes were only statistically significant for axial rotation. Since freezing temperatures were constant for each cycle, it seems reasonable that the largest stiffness changes would occur the first time that any micro-structural damage caused by ice crystal formation within the tissue was subjected to loading, i.e. between the first and second cycles[19].

The findings of both tests show that bending movements are more susceptible than axial rotation to testing environments. We speculate that this is related to the stabilising ligaments of the spine. These are mainly oriented in a longitudinal or oblique fashion with the thickest ligaments oriented in a longitudinal direction. As a result any change in stiffness of these ligaments will affect the bending movements more than axial rotation.[20, 21]

There are several limitations to this study. Firstly, a fixed sequence of loading directions was used. All specimens were tested in the order of flexion/extension, lateral bending followed by axial rotation. It would be of value to ascertain in future whether or not the loading sequence has any significant effect on the measured stiffness, or whether one of the loading directions in isolation is responsible for most of the observed stiffness change. Secondly, the testing was conducted without axial loading of the spine segments. Although axial loading is significant in the lumbar spine, it is unclear as to its significance in the thoracic spine, especially in a quadruped animal model, and therefore was not included in this study. Thirdly, rotation and moment measurements were recorded in the primary loading direction only in this study. Therefore the values of any coupled moments as well as their significance, remain unknown. Fourthly, the stiffness was calculated over the region of the moment vs rotation curve between 0.5 and 1Nm. The choice of region would be expected to have some effect on the calculated stiffness value. However as this study was evaluating the change in stiffness with the intervention, and the same moment range was used consistently throughout, this limitation is likely to have an insignificant effect on the results presented here. Also the segments were not subjected to an axial load, this was done to allow us to compare our results with previous studies using the same testing protocol[6, 18]. Finally all specimens were all frozen prior to any testing. As a result no fresh specimen testing was conducted and could not be included in this study.

Conclusion:

In vitro biomechanical testing of immature bovine thoracic spine segments can be performed up to 200 cycles without significant changes in stiffness. However, when testing protocols require greater than 200 cycles, or when repeated freeze-thaw cycles are involved, it is important to account for the effect of cumulative load cycles especially in flexion/extension or lateral bending. We would also recommend designing the study so as to allow the biomechanical testing to be completed in a single session to minimize the effect of repeated freeze-thaw cycles.

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